

CLAIM AMENDMENTS

This listing of claims replaces all prior versions and listings of claims in the application:

1. (original) A method for inserting a nucleic acid fragment into a circular vector, which comprises:

(a) stably joining an insertion end of a nucleic acid fragment with an insertion end of a linearized vector at a first nucleic acid concentration under conditions favoring intermolecular joining, to form a linear vector-insert concatemer;

(b) melting hybridized cohesive circularization ends in said vector-insert concatemer to form a linear vector-insert monomer having single-stranded cohesive circularization ends; and

(c) reannealing said single-stranded cohesive circularization ends at a second nucleic acid concentration under conditions favoring circularization to form a circularized vector containing a nucleic acid insert;

wherein said second nucleic acid concentration is more dilute than said first nucleic acid concentration and wherein said cohesive circularization ends are between about 8 and about 50 nucleotides in length.

2. (currently amended) A method for inserting a nucleic acid fragment into a circular vector, which comprises:

(a) stably joining an insertion end of a nucleic acid fragment with an insertion end of a linearized vector at a first nucleic acid concentration under conditions favoring intermolecular joining, to form a linear vector-insert construct with complementary circularization ends, wherein one or both circularization ends of said vector-insert construct (1) are attached to an enzyme or enzyme complex capable of covalently joining DNA ends, and (2) are blocked from covalent joining;

(b) unblocking said circularization ends of said vector-insert construct; and

(c) joining said circularization ends of said insert-vector construct at a second nucleic acid concentration in an intramolecular reaction mediated by said enzyme or enzyme complex under conditions favoring circularization, to form a circularized vector containing a nucleic acid insert;

wherein said second nucleic acid concentration is more dilute than said first nucleic acid concentration ~~circularization~~.

3. (original) The method of Claim 2 wherein:

said enzyme or enzyme complex is a site-specific topoisomerase that is covalently linked through a 3' phosphate to a circularization end;

one or both circularization ends are blocked from covalent joining by 5' phosphates;

said unblocking is achieved by removing said 5' phosphates from said circularization ends; and

said site-specific topoisomerase does not substantially covalently join said circularization ends of said vector-insert construct until the 5'- phosphates are removed from said circularization ends.

4. (currently amended) The method of Claim 1 wherein said linearized vector comprises two vector parts, each vector part having:

(i) ~~a cohesive circularization end which can hybridize to a complementary cohesive circularization end of the second vector part~~ an insertion end; and

(ii) ~~an insertion end~~ a cohesive circularization end which can hybridize to a complementary cohesive circularization end of the second vector part.

5. (currently amended) The method of Claim 1 wherein said linearized vector comprises an insertion end and a cohesive circularization end and wherein said nucleic acid fragment comprises:

(i) ~~a complementary~~ an insertion end that is complementary to said insertion end of said linearized vector; and

(ii) ~~a complementary~~ cohesive circularization end that is complementary to said cohesive circularization end of said linearized vector.

6. (original) The method of Claim 1 wherein:

said vector has a recognition site for an enzyme or enzyme complex which creates a first nick in one strand which is about 8 to about 50 nucleotides from a second nick in the other strand;

after said joining, said method further comprises nicking said vector-insert concatemer with said enzyme or enzyme complex to produce cohesive circularization ends;

said nicking is not accompanied by packaging into phage particles; and said recognition site is at least about 15 nucleotides in length.

7. (original) The method of Claim 2 wherein said linearized vector comprises two vector parts, each vector part having an insertion end and a circularization end, and wherein one or both of said circularization ends (1) are attached to an enzyme or enzyme complex capable of covalently joining DNA ends, and (2) are blocked from covalent joining.

8. (currently amended) The method of Claim 2 wherein:

said linearized vector comprises an insertion end and a circularization end;

said nucleic acid fragment comprises ~~a complementary insertion end and a complementary circularization end~~ an insertion end that is complementary to said insertion end of said linearized vector and a cohesive circularization end that is complementary to said cohesive circularization end of said linearized vector;

either said circularization end or said complementary circularization end is attached to an enzyme or enzyme complex capable of covalent joining DNA ends; and

both said circularization end and said complementary circularization end are blocked from covalent joining.

9. (currently amended) The method of Claim 1 ~~wherein said cohesive circularization ends are formed~~ further comprising the step of forming said cohesive circularization ends by the use of an enzyme or enzyme complex which creates a first nick in one strand which is about 8 to about 50 nucleotides from a second nick in the other strand.
10. (currently amended) The method of Claim 1 ~~wherein said cohesive circularization ends are formed~~ further comprising the step of forming said cohesive circularization ends by the use of a terminase of a bacteriophage or virus, but wherein said vector-insert monomer is not packaged into a phage particle.
11. (currently amended) The method of Claim 1 ~~wherein said cohesive circularization ends are formed~~ further comprising the step of forming said cohesive circularization ends by ligation of oligonucleotide adapters, tailing with terminal transferase, digestion with an exonuclease, digestion with a DNA polymerase possessing proofreading activity, or removal of uracil residues by uracil DNA glycosylase after polymerase chain reaction using dUMP-containing primers.
12. (original) The method of Claim 1 wherein said cohesive circularization ends comprise nucleotide analogs.
13. (original) The method of Claim 1 or 2 wherein said joining is mediated by ligase and wherein ligase does not substantially covalently join said circularization ends.
14. (original) The method of Claim 1 or 2 wherein said joining is mediated by a site-specific topoisomerase covalently linked to said insertion end of said linearized vector or said insertion end of said nucleic acid fragment.
15. (original) The method of Claim 1 or 2 wherein said joining is mediated by Vaccinia virus topoisomerase I or a Vaccinia virus topoisomerase I fusion protein covalently linked to said insertion end of said linearized vector or said insertion end of said nucleic acid fragment.

16. (currently amended) The method of Claim 1 or 2 wherein at least one of said insertion ends or at least one of said circularization ends comprises a blunt end covalently linked to a site-specific topoisomerase and wherein said ~~blunt end is prepared by~~ method further comprises preparing said blunt end by:

- (a) creating a nick in a DNA strand that is exactly opposite to a topoisomerase cleavage site in the complementary DNA strand; and
- (b) cleaving with said site-specific topoisomerase at said topoisomerase cleavage site to produce a blunt end.

17. (currently amended) The method of Claim 1 or 2 wherein at least one of said insertion ends or at least one of said circularization ends comprises a 3' overhang covalently linked to a site-specific topoisomerase and wherein said ~~3' overhang is prepared by~~ method further comprises preparing said 3' overhang by:

- (a) creating a nick in a DNA strand that is located one or more nucleotides in the 3' direction from a position exactly opposite to a topoisomerase cleavage site in the complementary DNA strand; and
- (b) cleaving with said site-specific topoisomerase at said topoisomerase cleavage site to produce a 3' overhang.

18. (original) The method of Claim 2 wherein said joining is mediated by annealing a cohesive insertion end of said linearized vector to a complementary cohesive insertion end of said nucleic acid fragment, wherein each of said cohesive insertion ends is between about 8 to about 50 nucleotides in length.

19. (original) The method of Claim 1 wherein said reannealing is performed at a salt concentration which is higher than the salt concentration used for said melting.

20. (original) The method of Claim 1 wherein said reannealing is performed at a salt concentration which is between about 100 mM and about 7.5 M.
21. (original) The method of Claim 1 wherein said reannealing is performed at about 50°C to about 85°C.
22. (previously amended) The method of Claim 1 or 2 wherein said nucleic acid fragment is selected from the group consisting of eukaryotic, prokaryotic, viral, and bacteriophage genomic DNA, cDNA, cDNA:RNA hybrid, polymerase chain reaction product and vector DNA.
23. (previously amended) The method of Claim 1 or 2 wherein said nucleic acid fragment is present at 10^{-21} to 10^{-14} mole.
24. (original) The method of Claim 1 or 2 wherein said first nucleic acid concentration comprises a molar ratio of linearized vector to nucleic acid fragment which is about 10:1 to about 100,000,000:1.
25. (original) The method of claim 1 or 2 wherein said conditions favoring intermolecular joining are macromolecular crowding conditions.
26. (original) The method of Claim 1 or 2 wherein said second nucleic acid concentration is less than one tenth of said first nucleic acid concentration.
27. (original) The method of Claim 1 or 2 wherein the efficiency of insertion of said nucleic acid fragment into said circular vector is at least about 95%.
28. (original) The method of Claim 1 or 2 wherein the efficiency of forming a circularized vector containing only one nucleic acid insert is at least about 95%.

29. (original) The method of Claim 1 or 2 wherein the efficiency of forming a circularized vector containing an insert is substantially the same over a range of insert sizes varying from about 20 base pairs to about 20,000 base pairs.

30. (original) The method of Claim 1 or 2 wherein said nucleic acid fragment is between 20 base pairs and 100,000 base pairs in length.

31-41. (canceled)

42. (original) A linearized vector comprising an origin of replication, two blunt or sticky ends, and two cohesive ends, wherein:

said cohesive ends are between about 8 and about 50 nucleotides in length;
each of said blunt or sticky ends is covalently linked to a site-specific topoisomerase; and
each of said blunt or sticky ends has a 5'-phosphate.

43. (original) A linearized vector comprising an origin of replication, a blunt or sticky end covalently linked to a site-specific topoisomerase, and a cohesive end, wherein said cohesive end is between about 8 and about 50 nucleotides in length.

44. (original) A kit comprising a first compartment containing the linearized vector of Claim 42 or 43.

45. (original) The kit of Claim 44 which further comprises:

a second compartment containing a buffer comprising polyethylene glycol of high molecular weight; and
a third compartment containing a buffer comprising a salt.

46. (original) A linearized vector comprising an origin of replication, two insertion ends, and two circularization ends wherein:

each of said circularization ends is located at least 15 base pairs from each of said insertion ends;

each of said insertion ends is covalently linked to a site-specific topoisomerase;

one or both of said circularization ends are covalently linked to a site-specific topoisomerase; and

each of said insertion ends and each of said circularization ends has a 5'-phosphate.

47. (original) A kit comprising a first compartment containing the linearized vector of Claim

48. (original) A linearized vector comprising an origin of replication, a bacteriophage or virus cos site, and two insertion ends covalently linked to a site-specific topoisomerase.

49. (original) A kit comprising a first compartment containing the linearized vector of Claim

50. (original) The kit of Claim 49 which further comprises:

a second compartment containing a buffer comprising high molecular weight polyethylene glycol;

a third compartment containing a terminase; and

a fourth compartment containing a buffer comprising a salt.

51-68. (canceled)